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בקשה לפטנט  
Application for Patent

אני, (שם, המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)  
I (Name and address of applicant, and in case of body corporate - place of incorporation)

YISSUM RESEARCH DEVELOPMENT COMPANY OF THE  
HEBREW UNIVERSITY OF JERUSALEM  
Jabotinsky 46, Jerusalem

יישום חברה לפיתוח המחקר של  
האוניברסיטה העברית בירושלים  
חברה ישראלית בערבון מוגבל  
ז'בוטינסקי 46, ירושלים

Inventors: Iris Bahir  
Shmuel Ben-Sasson

ממציאים: - איריס בהיר  
שמואל בן-ששון

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in invention the title of which is  
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ששמה הוא  
(בעברית)

שיטה לייצור מגוון זני צמחים

(English)

(באנגלית)

A METHOD FOR GENERATING PLANT DIVERSITY

hereby apply for a patent to be granted to me in respect thereof.

מבקש בזאת כי ינתן עליה פטנט

• בקשת חלוקה - Application of Division		• בקשת פטנט מוסף - Application for Patent Addition		דרישת דין קדימה Priority Claim		
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המען למסירת מסמכים בישראל Address for Service in Israel LUZZATTO & LUZZATTO P.O. Box 5352 Beer-Sheva 84152						
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**שיטה לייצור מגוון זני צמחים**

**A METHOD FOR GENERATING PLANT DIVERSITY**

## Field of the Invention

The present invention relates to the field of genetic engineering of plants. More specifically, the present invention provides a method for the generation of new plant varieties, using micro-satellite sequences as a tool to achieve plant diversity.

## Background of the Invention

Until now, conventional genetic engineering of plants target specific genes that are advantageous from a commercial point of view. For example, generation of transgenic plants that are resistant to herbicides or plants that produce fruits with a protein of higher nutritional value [McLaren J.S. (1998) *Pest Outlook* 12:36-41]. This is usually done via the introduction of foreign genetic elements into the plant genome [Kumar S. and Fladung M. (2001) *Trends in Plant Science* 6:155-9]. However, many of the important traits of plants are quantitative in nature and depend on the level of expression of multiple genes.

Micro-satellites (MSs) are repetitive DNA sequences typified by a monotonous repetition of short DNA sequences of between one to about ten nucleotides in length of the repeating unit [Moxon E.R. and Wills C. (1999) *Scientific American* 280(1):72-7]. The following DNA stretches are examples of MS sequences, (A)<sub>n</sub>, (CA)<sub>n</sub>, (CAG)<sub>n</sub>, (GATA)<sub>n</sub>, (TAGAAA)<sub>n</sub>, where n can vary between 3 to about 100 and the total length of the MS varies between about 10 to about 200 base-pairs (bp) in length. MS sequences may contain errors in the form of a base change or a missing nucleotide (i.e. a point mutation or a frame-shift) but still are considered hereby as genuine MS-like sequences. MSs are generally considered as "junk DNA", a remnant of Darwinian evolution that the genome could not get rid of.

The present invention is based on the premise that MS sequences have a regulatory role in the plant genome. Thus, the introduction of MS sequences

into the plant genome might result on a profound effect on the pattern of gene expression in the manipulated plant. The present invention describes a method whereby the incorporation of selected MS sequences into the genome of a large number of plant cells, and the growth of individual plants out of these individual cells, generates a plethora of new plant varieties.

These and other objects of the present invention will become apparent as the description proceeds.

### **Summary of the Invention**

In a first aspect, the present invention relates to a method of generation of genetic diversity among plants of the same species via the incorporation of exogenous micro-satellite (MS) sequences into the plant genome, wherein said method comprises the steps of:

- (a) Obtaining MS-like DNA fragments;
- (b) Introduction of the DNA into plant cells;
- (c) Selection of the plant cells containing the exogenous DNA;
- (d) Cultivation of the plants grown from the selected cells under suitable conditions.

Optionally, the MS-like DNA fragments obtained in step (a) may be ligated into suitable vectors and then introduced into plant cells.

In a preferable embodiment, the MS-like DNA fragment (also referred to as exogenous MS, or exogenous DNA) is introduced into plant cells concomitantly with a selective marker. Said selective marker may be a gene that confers resistance to an antibiotic, a herbicide or a metabolic inhibitor.

In one embodiment of the method of the invention, the MS-like DNA fragment comprises a monotonous repeat of one to six nucleotides and is at least twelve nucleotides in length, wherein said repeat is any one of A/T, AT/TA, AG/CT, AAG/CTT, CGG/CCG, ATCG/CGAT or AAAT/ATTT.

In another embodiment, the MS-like DNA fragment comprises a sequence that is at least 70% homologous to the above-mentioned monotonous repeat.

In a further embodiment of the method of the invention, the synthetic MS-like DNA fragment further includes in tandem a unique identifiable sequence that enables specific tagging of the incorporated DNA.

In a yet further embodiment of the invention, the synthetic MS-like DNA fragment is introduced into individual plant cells. Alternatively, the synthetic MS-like DNA fragment is introduced into any one of a plant embryo, a plant tissue or callus, or a leaf, which are then subsequently disintegrated into individual plant cells. Said individual cells are cultivated to give rise to individual plants.

In a further further embodiment of the method of the invention, the exogenous DNA is obtained via synthesis or cloning. In addition, the exogenous DNA may be produced by the ligation of several DNA pieces.

In a last embodiment of the method of the invention, the DNA may be introduced via any one of electroporation, chemical or mechanical means, or liposomes. Said DNA may be either naked, or it may be within a construct, and be introduced into the plant genome by a genetic vehicle such as a plasmid or a viral vector.

In another aspect, the invention refers to the use of MS-like DNA fragments as a tool for the generation of new plant varieties, or for the generation of any one of cells, seeds or progeny of said plants.

In a further aspect, the invention provides a plant variety produced by the method of obtaining and introducing MS-like DNA fragments into plant cells, selecting the plant cells containing the exogenous DNA, and cultivating the plants grown from said selected cells under suitable conditions.

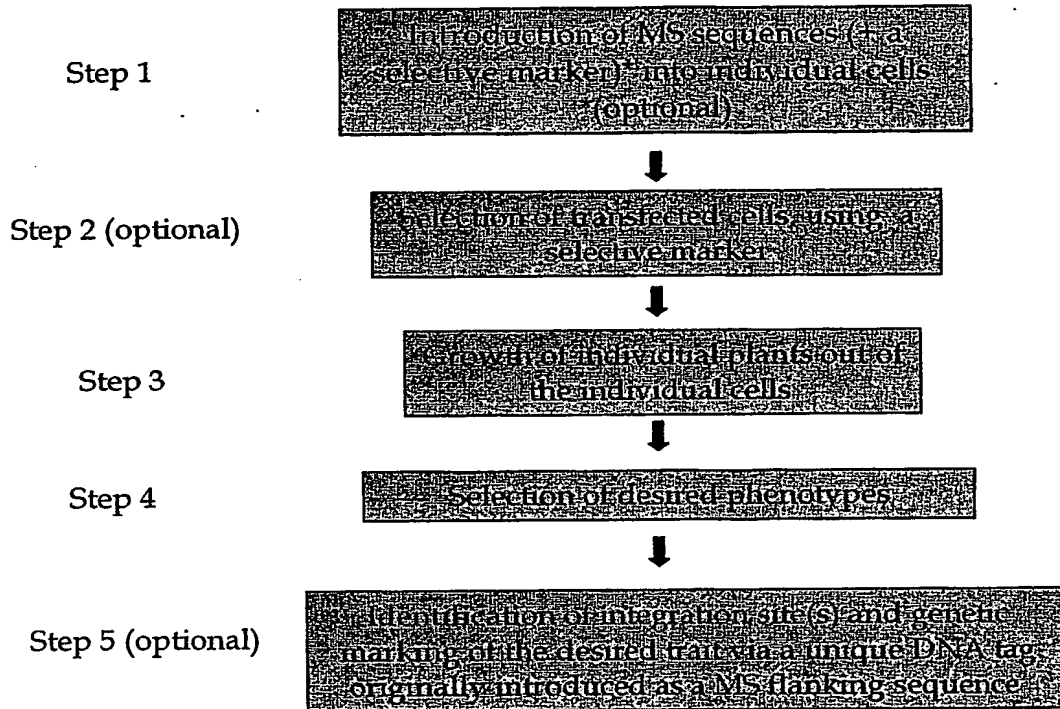
A further aspect of the invention refers to a plant variety whose genome has been modified by the method described in the present invention.

Lastly, the present invention provides a new plant variety generated by the introduction of MS-like DNA fragments into its genome, and cells, seeds and progeny thereof.

## Flowchart summarizing the invention

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## Generation of Phenotypic Diversity in Plants

**Detailed Description**

In search for a method for generating plant diversity, the present inventors were based on the premise that MS sequences have a regulatory role in the plant genome, and that consequently, insertion of such DNA sequences into plant cells will result in diversified phenotype.

Thus, in a first aspect, the present invention provides a method for the generation of genetic diversity among plants of the same species, via the



incorporation of exogenous micro-satellite (MS) sequences into the plant genome, wherein said method comprises the steps of:

- (a) Obtaining MS-like DNA fragments;
- (b) Introduction of the DNA into plant cells, preferably concomitantly with a selection marker;
- (c) Selection of the plant cells containing the exogenous DNA;
- (d) Cultivation of the plants grown from the selected cells.

Optionally the DNA fragments may be ligated to suitable vectors and then introduced into the plant cells.

In one embodiment, the MS sequence utilized in the method of the invention comprises a monotonous repeat of one to six nucleotides, at least twelve nucleotides long, with maximum length of 10,000 nucleotides. Preferably, said repeat is any one of A/T, AT/TA, AG/CT, AAG/CTT, CGG/CCG, ATCG/CGAT or AAAT/ATTT. Alternatively, the MS-like DNA fragment comprises a sequence that is at least 70% homologous to said monotonous repeat.

In another embodiment of the invention, the exogenous MS is introduced concomitantly with a selective marker, wherein preferably said selective marker is a gene that confers resistance to an antibiotic, a herbicide, or a metabolic inhibitor. Alternatively, the selective marker can be a gene that highlights the transfected cells such as the gene for green fluorescent protein (GFP). Thus, the MS-like DNA fragments and the selection marker will be introduced into the plant or plant cells by co-transfection.

Preferably the synthetic MS sequence may further include a unique identifiable sequence in tandem, which will enable specific tagging of the incorporated DNA and identification of the site of integration. This

embodiment is particularly important for the unequivocal identification of the transgenic plants.

In a further embodiment of the invention, the synthetic MS sequence is introduced into individual plant cells. Alternatively, the synthetic MS sequence is introduced into any one of a plant embryo, a plant tissue, a callus, leaves, or any plant part where it is possible to introduce DNA. When the MS sequence is introduced into a plant cell which is part of a multi-cellular tissue, said tissue will be subsequently disintegrated into individual plant cells, to obtain a single cell suspension. The cells containing the introduced DNA will be selected, and then cultivated under suitable conditions to give rise to individual plants.

The MS sequences to be incorporated into the plant genome may be introduced via any technique known to the artisan for the introduction of DNA sequences into a genome, such as electroporation, mechanical means like the gene gun (also known as particle bombardment), chemical means such as polyethylene glycol, or by the use of liposomes, with or without being carried by vectors. Alternatively, the DNA may be introduced into protoplasts.

The MS sequences may be introduced into the plant genome as fragments. Alternatively, these sequences may first be ligated. Such a ligated construct may be further incorporated into a genetic vehicle such as a plasmid or a viral vector, and then introduced into the plant genome.

The exogenous MS sequences to be used in the method of the invention are to be obtained via synthesis or cloning, i.e., said MS-like DNA fragments may be synthetically made or they may be isolated from the genome of a plant cell.

In an additional embodiment of the invention, the exogenous DNA construct to be introduced for the generation of plant diversity may be produced by the ligation of the MS-like fragments with a marker DNA, a tagging sequence and a vehicle, or any combination thereof. Thus, the MS-like fragment may be introduced as naked DNA or as part of a larger structure, as for example in a DNA construct.

The cultivation step of the method of the present invention may itself be selective. For example, plants may be cultivated under stressing conditions such as high salinity, for selection of salt-resistant plants, or be treated with herbicides, for selection of herbicide-resistant plants. Thus, the invention also encompasses the selection of plants with desired traits.

In a third aspect, the invention provides the use of MS sequences as a tool for the generation of new plant varieties and cells, seeds or progeny thereof. It is understood that the method of the invention will modify the genome of a very large number of plant cells at once. Since insertion of said MS sequences will occur at random, a very large number of new phenotypes will be generated by the method of the invention. Within such large choice of new phenotypes, beneficiary traits will be abundant.

Another aspect of the invention is a plant variety produced by the method of the invention. The method of the invention shall enable the establishment of a diversity of stable phenotypes, from which new plant varieties may be developed.

A further aspect of the invention provides a plant variety whose genome has been modified by the method of the invention.

Lastly, the invention provides a new plant variety generated by the introduction of MS-like DNA fragments into its genome and cells, seeds and progeny thereof.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

## Examples

### Experimental Procedures

#### Synthesis of DNA fragments

DNA fragments can be synthetically made using commercially available DNA synthesizers [for details see: *Current Protocols in Molecular Biology*, Editors: Ausubel FM et al. (2002) Published by John Wiley & Sons], or via the conventional polymerase chain reaction (PCR) method [Ausubel et al. (2002) *ibid.*] The different DNA fragments (e.g the MS and the tagging sequence; for more details see the above flowchart) are then ligated by methods known to those versed in the art.

#### Introduction of DNA fragments into plant cells

A DNA can be transferred into plant cells by applying a high voltage, a method known as electroporation [D'Halluin K. et al. (1992) *Plant Cell* 4:1495-1505], or by a temporary mechanical disruption of the cell membrane [Taylor NJ and Fauquet CM. (2002) *DNA Cell Biol.* 21:963-77]. This last method is conventionally known as the gene gun, and involves coating miniature metal beads (gold or tungsten, around 1  $\mu\text{m}$  in diameter and less) with the exogenous DNA and forcing them through the cell membrane using a high pressure device, known as particle bombardment gun. Yet another way to introduce exogenous DNA into the plant genome is via genetic vectors such as plasmids or viral genomes. Examples of such genetic vectors are *Agrobacterium*-mediated transformation or the infection of cells with recombinant viruses such as the tobacco mosaic virus (TMV) or the cowpea mosaic virus (CPMV).

#### Example 1: Generation of new plant varieties

The incorporation of MS sequences into new sites within the plant genome enables the generation of a variety of plant strains with a broad diversity of characteristics that is genetically inherited. The desired strain shall then be

selected according to preferred properties. For example, plants can be selected for flower or fruit size, plant height, resistance to herbicides, endurance to salinity or heat, or any other feature that might be advantageous for different purposes. Thus, the inventors present a novel approach for genetic engineering of plants and a method to its implementation.

DNA fragments can be introduced into plant cells through different ways, mainly via electroporation, by chemical means or by mechanical disruption of the cell membrane. The DNA fragments may be carried by genetic vectors or not, and the site of integration shall be random.

Once introduced into the cell nucleus, DNA fragments become incorporated into the plant genome. For reasons of simplicity, the term transfection is herein used to include all different methods of introduction of exogenous DNA into the plant genome.

In order to enhance the selection of cells that actually incorporated the transfected DNA, it is advantageous to introduce a selective marker concomitantly with the desired DNA fragment. This can be achieved by co-transfecting the cells with a genetic element that confers resistance to a toxic agent, like a herbicide or an antibiotic, that can kill non-protected cells who did not accept the exogenous DNA. For example, such a selection marker can be a gene for an enzyme that degrades the said herbicide or antibiotic or clones that highlight the transfected cells. Clones of such genes are well known to those versed in the art [for example see Zhang CL. et al. (2001) *Mol. Biotechnol.* 17:109-17 and references therein]) and are commercially available. In addition, the introduced MS sequence can be flanked by a unique DNA sequence in order to tag the exogenously introduced MS. This shall enable an unequivocal identification of the site(s) of integration of the MS in the plant genome and the identification of a novel trait.

Plant cells can be transfected with exogenous DNA while part of a plant embryo, plant tissue like a leaf, a wound tissue or non-differentiated callus, or can be transfected while in single cell suspension or as protoplasts (plant cells whose cell-wall was dissolved). Following DNA transfection, selection of the modified cells is made at the single cell level by using an incorporated selectable marker screening procedure, which is then followed by development into individual plants .

Thus, the inventors hereby specify the incorporation of selected MS sequences into the genome of a large number of plant cells and the growth of individual plants out of these individual cells, by methods known to those versed in the art. Once integrated into the plant genome, this new pattern of MS distribution should be genetically stable and heritable.

The exact nature of the ensuing phenotypic change cannot be predicted in advance due to sporadic sites of MS integration and variability in copy number, unique to each transfected plant cell. Nevertheless, it should lead to the creation of a broad spectrum of phenotypes through the integration of MS sequences in different sites in the genome of different cells, occurred at random. This, in turn, will open the way for the generation of genetically defined strains of desirable phenotypes, through the selection of a desired trait, like for example plant size, fruit size, flower shape, resistance to salinity or heat, amongst others.

**Claims:**

1. A method of generation of genetic diversity among plants of the same species via the incorporation of exogenous micro-satellite (MS) sequences into the plant genome, wherein said method comprises the following steps:
  - (a) Obtaining MS-like DNA fragments;
  - (b) Introduction of the DNA into plant cells;
  - (c) Selection of the plant cells containing the exogenous DNA;
  - (d) Cultivation of the plants grown from the selected cells under suitable conditions.
2. The method of claim 1, wherein the MS-like DNA fragment comprises a monotonous repeat of one to six nucleotides and is at least twelve nucleotides in length.
3. The method of claim 1, wherein the MS-like DNA fragment comprises a sequence that is at least 70% homologous to a monotonous repeat of one to six nucleotides and is at least twelve nucleotides in length.
4. The method of claims 2 and 3, wherein said repeat is any one of A/T, AT/TA, AG/CT, AAG/CTT, CGG/CCG, ATCG/CGAT or AAAT/ATTT
5. The method of any one of the preceding claims, wherein optionally the MS-like DNA fragments obtained in step (a) are ligated into suitable vectors and then proceed to step (b).
6. The method of any one of the previous claims, wherein the exogenous MS is preferably introduced concomitantly with a selective marker.



7. The method of claim 6, wherein the selective marker is a gene that confers resistance to an antibiotic, a herbicide or a metabolic inhibitor.
8. The method of claims 1 to 7, wherein the synthetic MS-like DNA fragment further includes in tandem a unique identifiable sequence that enables specific tagging of the incorporated DNA.
9. The method of any one of claims 1 to 8, wherein the synthetic MS-like DNA fragment is introduced into individual plant cells.
10. The method of any one of claims 1 to 8, wherein the synthetic MS-like DNA fragment is introduced into any one of a plant embryo, a plant tissue or callus, or a leaf, which are then subsequently disintegrated into individual plant cells.
11. The method of any one of claims 9 or 10, wherein the individual cells are cultivated to give rise to individual plants.
12. The method of any one of claims 1 to 11, wherein the DNA is introduced via any one of electroporation, chemical, mechanical means or liposomes.
13. The method of any one of claims 1 to 12, wherein the DNA is introduced by a genetic vehicle such as a plasmid or a viral vector.
14. The method of any one of the previous claims, wherein the exogenous DNA is obtained via synthesis or cloning.

15. The method of any one of the preceding claims, wherein the exogenous DNA is produced by the ligation of several DNA pieces.
16. Use of MS-like DNA fragments as a tool for the generation of new plant varieties.
17. The use of claim 16, for the generation of any one of cells, seeds or progeny of said plants.
18. A plant variety produced by the method of any one of claims 1 to 15.
19. A plant variety whose genome has been modified by the method defined in any one of claims 1 to 15.
20. A new plant variety generated by the introduction of MS-like DNA fragments into its genome, and cells, seeds and progeny thereof.

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